

# The response of $\gamma$ vitamin E to varying dosages of $\alpha$ vitamin E plus vitamin C

Absalon D. Gutierrez, Daniela Gonzalez de Serna, Irina Robinson, David S. Schade\*

*University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA*

Received 17 July 2007; accepted 20 November 2008

## Abstract

Vitamin E has been studied extensively in the prevention of atherosclerosis. Cross-sectional population studies as well as randomized controlled intervention trials have demonstrated conflicting results. A recent meta-analysis of these trials has emphasized the ineffectiveness of vitamin E in atherosclerosis prevention, with a possibility of harm at higher dosages. However, vitamin E has several isomers, with the  $\alpha$  form being available via dietary supplements and the  $\gamma$  form being available via dietary foodstuffs. The  $\gamma$  form of vitamin E demonstrates several superior properties (such as trapping reactive nitrogen species and detoxifying nitrogen dioxide) compared with  $\alpha$  vitamin E. All clinical trials have used the  $\alpha$  isomer, with little concern that this isomer of vitamin E may actually suppress the  $\gamma$  isomer of vitamin E. We undertook a dose-response study in volunteers with type 2 diabetes mellitus to include all the dosages of  $\alpha$  vitamin E that have been used in cardiovascular prevention trials to determine the effect of  $\alpha$  vitamin E on  $\gamma$  vitamin E. We also assessed the effect of  $\alpha$  vitamin E on several traditional markers of atherosclerotic risk. We added vitamin C to the vitamin E because several clinical trials included this vitamin to enhance the antioxidant effects of  $\alpha$  vitamin E. Volunteers received, in randomized order for a 2-week period, one of the following vitamin dosage arms: (1) no vitamins, (2) low-dose supplemental vitamins E plus C, (3) medium-dose supplemental vitamins E plus C, and (4) high-dose supplemental vitamins E plus C. Blood levels of both  $\alpha$  and  $\gamma$  vitamin E were measured as well as surrogate markers of oxidative stress, hypercoagulation, and inflammation during a high-fat atherogenic meal (to increase the ambient oxidative stress level during the study). The results demonstrate that  $\alpha$  vitamin E levels increased in proportion to the dose administered. However, at every dose of  $\alpha$  vitamin E,  $\gamma$  vitamin E concentration was significantly suppressed. No beneficial changes in surrogate markers of atherosclerosis were observed, consistent with the negative results of prospective clinical trials using  $\alpha$  vitamin E. Our results suggest that all prospective cardiovascular clinical trials that used vitamin E supplementation actually suppressed the beneficial antioxidant  $\gamma$  isomer of vitamin E. No beneficial effects on several potential cardiovascular risk factors were observed, even when the vitamin E was supplemented with vitamin C. If a standardized preparation of  $\gamma$  vitamin E (without the  $\alpha$  isomer) becomes available, the effects of  $\gamma$  vitamin E on atherosclerotic risk will warrant additional studies.

© 2009 Elsevier Inc. All rights reserved.

## 1. Introduction

The antioxidant vitamin E has been a major subject of controversy in the prevention of atherosclerosis. The initial basic science studies suggested that vitamin E had beneficial effects on several different stages of the atherosclerotic process [1]. Vitamin E has not only antioxidant properties, but also nonantioxidant effects, including modulation of

signal transduction pathways [2]. This was followed by observational, cross-sectional studies in patients with no initial coronary artery disease, which also suggested that vitamin E supplementation lowered the risk of major coronary heart disease (CHD) [3–5]. However, subsequent large randomized controlled trials have shown that vitamin E supplementation had no clear benefit in the primary or secondary prevention of cardiovascular disease, with some trials suggesting harmful effects [6]. The recently published Women's Health Study found that 600 IU of vitamin E, given on alternate days for 10 years, demonstrated no overall benefit in the prevention of major cardiovascular events [7]. The Heart Outcomes Prevention Evaluation–The Ongoing Outcomes (HOPE-TOO) trial concluded that long-term

ClinicalTrials.gov identifier: [NCT00362518](https://clinicaltrials.gov/ct2/show/study/NCT00362518).

\* Corresponding author. Endocrinology and Metabolism, Endocrinology MSC10 5550, 1 University of New Mexico, Albuquerque, NM 87131-0001, USA. Tel.: +505 272 4657; fax: +505 272 5155.

E-mail address: [dschade@salud.unm.edu](mailto:dschade@salud.unm.edu) (D.S. Schade).

vitamin E supplementation (400 IU/d for 10 years) did not prevent cardiovascular events and may have actually increased the risk of heart failure [8,9].

Of the clinical trials showing benefit, most of them studied patients with evidence of increased oxidative stress and, in addition, gave vitamin C supplements with the vitamin E. Vitamin C, a water-soluble antioxidant, has been shown to regenerate vitamin E (a lipid-soluble antioxidant) [10,11]. In patients with increased oxidative stress (ie, patients with conditions such as diabetes mellitus, end-stage renal disease, coronary artery disease, and/or hypercholesterolemia), vitamin C restores endothelium-dependent artery vasodilation and may reverse endothelial dysfunction in peripheral arteries [10–13]. Three recent trials using high dosages of both vitamins E and C together, in populations with high oxidative stress, have shown a delay of coronary arteriosclerosis when compared with placebo [14–17]. Supplementation of both vitamins, in individuals with increased oxidative stress, may therefore be more effective than either vitamin taken alone.

All of the above-cited clinical trials used the  $\alpha$  tocopherol isomer form of vitamin E ( $\alpha$  vitamin E). This is the composition of vitamin E sold in over-the-counter supplements. However, there has been growing interest in another isomer of vitamin E, specifically  $\gamma$  tocopherol ( $\gamma$  vitamin E), which is believed to play a distinct and beneficial role in atherosclerosis prevention [2]. Several studies have suggested that  $\alpha$  tocopherol supplementation may decrease serum levels of  $\gamma$  tocopherol in man [18,19]. However, no variable dose-response studies of the effects of  $\alpha$  tocopherol on  $\gamma$  tocopherol have been published; so the overall effects of  $\alpha$  tocopherol are unknown. Because a wide range of  $\alpha$  tocopherol dosages has been used in clinical trials, this information is critical to evaluating the effects of vitamin E on atherosclerotic risk.

In addition, surrogate markers of atherosclerosis provide a useful approach to understanding the effects of an intervention on different components of the atherosclerotic process, including oxidative stress, inflammation, and hypercoagulation. Therefore, it was the intent of this study to examine the potential benefits of  $\alpha$  tocopherol supplementation (plus vitamin C), or lack thereof, by administering different dosages of these vitamins to individuals with type 2 diabetes mellitus (a population with high oxidative surrogate markers of atherosclerosis).

## 2. Methods

### 2.1. Subjects

The study enrolled 12 adult subjects with well-controlled, non-insulin-requiring type 2 diabetes mellitus of more than 6 months' duration being treated with diet plus a maximum of 2 oral diabetes medications. Subjects were excluded if they had known cardiovascular, hepatic, or renal disease; uncontrolled hypertension ( $>140/90$  mm Hg); marked hyperlipidemia (serum low-density lipoprotein [LDL]  $>4.1$  mmol/L

or serum triglycerides  $>7.8$  mmol/L); a body mass index greater than  $40 \text{ kg/m}^2$ ; or a hemoglobin A<sub>1c</sub> greater than 9%. Eligible patients had a C-peptide stimulation test of at least 6 ng/mL after a Sustacal (Mead Johnson, Evansville, IN) challenge and normal hematologic, electrolyte, and hepatic laboratory results at screening. Other important exclusion criteria were cigarette smoking, gastroparesis, recent use (within 3 months) of antioxidant vitamin supplements, use of medications affecting coagulation and glucose homeostasis, pregnancy, and current malignancy. The study protocol was approved by the University of New Mexico Human Research Review Committee, and all patients gave written informed consent before study.

### 2.2. Study protocol

The study protocol was a randomized, crossover design. Assay technicians and the principal investigator were blinded to the study arms of the volunteers. Studies were performed in randomized sequence with vitamin E (RRR- $\alpha$ -tocopherol) and vitamin C supplementation. Each subject participated in 4 separate, day-long inpatient studies at the University of New Mexico General Clinical Research Center (GCRC). Each study represented 1 of the 4 arms of the overall study: no vitamins, low-dose vitamins ( $\alpha$  tocopherol = 200 IU, vitamin C = 250 mg), medium-dose vitamins ( $\alpha$  tocopherol = 400 IU, vitamin C = 500 mg), and high-dose vitamins ( $\alpha$  tocopherol = 800 IU, vitamin C = 1000 mg), each dose given once daily. Participants' usual diabetes medications were withheld only during the inpatient study day. Vitamin supplementation, determined by the arm of the study, was administered daily for 14 days before each inpatient study. Participants were contacted 3 times per week by telephone to encourage compliance with vitamin administration.

At the start of the study, an intravenous catheter was placed in an upper extremity; and participants took the same vitamins, C and E, that they had taken for the 2 weeks before the inpatient study. The patient was then fed a standardized American Diabetes Association composition recommended breakfast. After breakfast, participants were administered a continuous intravenous infusion of regular insulin for the purpose of achieving relative euglycemia. Upon achieving a blood glucose concentration between 80 and 120 mg/dL, the insulin was stopped, the catheter was removed, and a new catheter was placed in the contralateral arm. One hour later, the participant was fed an atherogenic high-fat test lunch equivalent to a McDonald's Big Mac meal. The meal was prepared in the GCRC metabolic kitchen and consisted of ground beef, American cheese, white hamburger bun, dill pickles, iceberg lettuce, regular thousand island dressing, onions, French fried potatoes (prepared in reheated 100% vegetable oil), and an ice-cream shake.

### 2.3. Study measurements

To determine circulating response to the vitamin E supplementation, plasma vitamin E (both  $\alpha$  tocopherol and

$\gamma$  tocopherol) was measured. Plasma levels of oxidized LDL (oxy-LDL), malondialdehyde (MDA), nonesterified fatty acids (NEFAs), plus erythrocyte intracellular glutathione peroxidase (GPx) activity were measured as surrogate markers of atherosclerotic risk and oxidative stress. Plasma levels of C-reactive protein (CRP), adiponectin, and interleukin-6 (IL-6) were assessed as surrogate markers of inflammation. Plasminogen activator inhibitor-1 (PAI-1) and fibrinogen activities were quantified as indices of hypercoagulation. Diabetes control was assessed with levels of fasting blood glucose and  $A_{1c}$ . Total cholesterol and triglycerides were measured for the purpose of calculating lipid-standardized vitamin E levels [20].

#### 2.4. Assays

All blood samples were processed immediately and frozen at  $-70^{\circ}\text{C}$  until assay. Plasma  $\alpha$  tocopherol and  $\gamma$  tocopherol were measured with high-performance liquid chromatography by ARUP Laboratories (Salt Lake City, UT) [21]. Plasma oxy-LDL levels were assayed with a solid-phase 2-site enzyme-linked immunosorbent assay (ELISA) kit from Mercodia (Uppsala, Sweden) [22]. Glutathione peroxidase activity in erythrocytes was assessed with a kit from Cayman Chemical (Ann Arbor, MI), which measured GPx activity through a coupled redox reaction using GPx and glutathione reductase [23,24]. A gas chromatography–mass spectrometry method was used to determine plasma free (unbound) MDA [25]. Serum NEFAs were measured with a kit from

Wako Diagnostics (Richmond, VA) [26]. High-sensitivity CRP (hsCRP) was determined by an immunoluminometric method from Diagnostic Products (Los Angeles, CA) [27]. Adiponectin was measured spectrophotometrically after being processed by a sandwich ELISA kit from LINCO Research (St Charles, MO) [28]. Interleukin-6 was measured with a highly sensitive quantitative sandwich ELISA technique and optical density measurements, with a kit from R&D Systems (Minneapolis, MN) [29]. The PAI-1 activity was measured using a Chromolize bioimmunoassay from Trinity Biotech (Bray, Ireland) [30]. Fibrinogen levels were assessed with the clot-rate method [31]. Plasma glucose was determined with the ACE glucose assay from Alfa Wassermann Diagnostic Technologies (West Caldwell, NJ) [32]. Plasma C-peptide and plasma insulin were each measured with chemiluminescent assays (Immunolite instruments) from Diagnostic Products [33,34]. Plasma triglycerides and cholesterol were assayed colorimetrically with ACE reagents from Alfa Wassermann [35,36].

#### 2.5. Statistical methods

The primary outcome variables assessed were the changes in plasma levels of both  $\alpha$  tocopherol and  $\gamma$  tocopherol. Secondary outcome variables were changes in plasma or serum concentrations of surrogate markers of atherosclerosis. Lipid-standardized tocopherol levels were calculated as previously described [20]. Tertiary variables were plasma levels of glucose, insulin, C-peptide, triglycerides, and

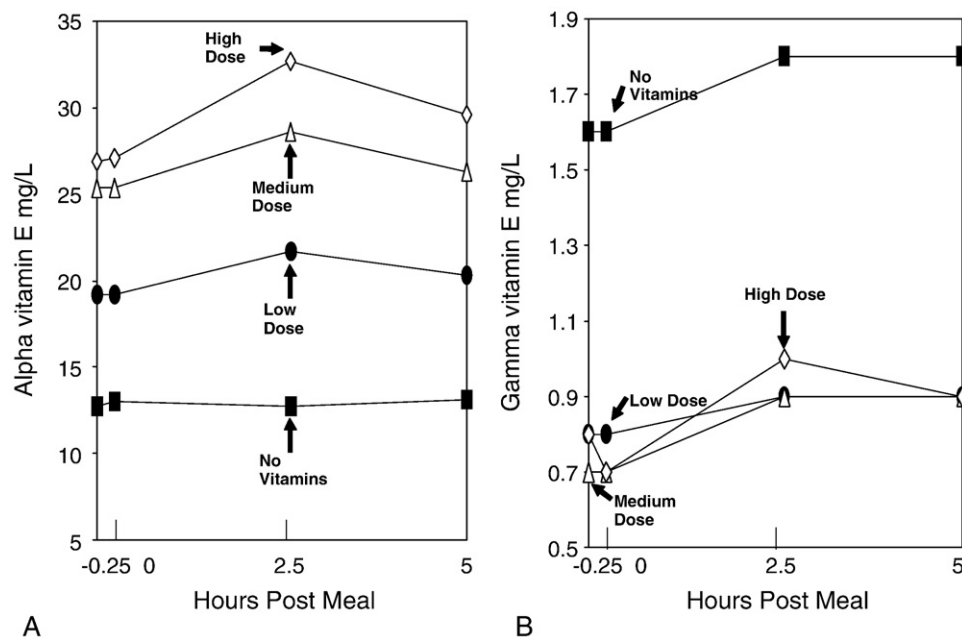


Fig. 1. Vitamin levels. Lipid-standardized plasma  $\alpha$  vitamin E (A) and  $\gamma$  vitamin E (B) excursions during the no-vitamin arm (black square), low-dose vitamin arm (black circle), medium-dose vitamin arm (open triangle), and high-dose vitamin arm (open diamond). All x-axis values (including the  $-0.25$  hour value) were obtained following breakfast and should be interpreted as relative to the lunch meal. The range for the SEM for lipid-standardized  $\alpha$  vitamin E at all data points was as follows: no-vitamin arm = 2.7 to 3.3, low-dose vitamin arm = 3.8 to 4.6, medium-dose vitamin arm = 6.0 to 7.3, and high-dose vitamin arm = 5.7 to 7.2. The range for the SEM for lipid-standardized  $\gamma$  vitamin E at all data points was as follows: no-vitamin arm = 0.9 to 1.1, low-dose vitamin arm = 0.2 to 0.5, medium-dose vitamin arm = 0.2 to 0.3, and high-dose vitamin arm = 0.2 to 0.3. At all doses of vitamin E supplementation,  $\gamma$  vitamin E was significantly suppressed by  $\alpha$  vitamin E ( $P < .01$ ).

cholesterol. Data were analyzed using the NCSS statistical program (NCSS, Kaysville, UT). Parameters were compared among the various groups by analysis of variance for repeated measures with post hoc Student *t* test for paired data where appropriate. Significance was defined by an  $\alpha$  of *P* less than .05. All data are reported as mean  $\pm$  SEM.

A power analysis using the data from Handelsman et al [37] indicated that a sample size of 8 subjects would achieve 86% power to detect a difference of  $-1.0$  between the null hypothesis mean of  $0.0$  and the alternative hypothesis mean of  $1.0$  with an estimated standard deviation of  $0.8$  and an  $\alpha$  of  $.05$  using a 2-sided *t* test.

### 3. Results

#### 3.1. Subject characteristics

Six men and 6 women (6 Hispanic, 5 non-Hispanic whites, 1 Native American) were studied. Mean  $\pm$  SEM age, body mass index, and duration of diabetes were  $53 \pm 4$  years,  $29 \pm 1$  kg/m<sup>2</sup>, and  $9 \pm 3$  years, respectively. The subjects had a mean  $\pm$  SEM hemoglobin A<sub>1c</sub> of  $6.2\% \pm 0.3\%$  and a mean fasting glucose of  $111 \pm 6$  mg/dL. The mean  $\pm$  SEM fasting lipid profile of the subjects consisted of a total cholesterol of  $158 \pm 9$  mg/dL, LDL cholesterol of  $77 \pm 6$  mg/dL, high-density lipoprotein cholesterol of  $45 \pm 2$  mg/dL, and triglycerides of  $153 \pm 20$  mg/dL.

#### 3.2. Plasma levels of vitamins E

Prebreakfast administration of vitamins (or no vitamins) showed a dose-dependent elevation in baseline levels of lipid-standardized  $\alpha$  tocopherol between the 4 different dosages (study arms). This led to 4 distinct, statistically different curves (baseline plus test meal) for the study arms ( $P < .0001$  for each compared with no vitamins) (Fig. 1, left panel). In addition, a dose-independent suppression in baseline and test meal  $\gamma$  tocopherol levels was observed when the low-, medium-, and high-dose  $\alpha$  tocopherol arms were compared with the no-vitamin arm. This resulted in a no-vitamin arm  $\gamma$  tocopherol curve that was significantly higher in comparison to the  $\gamma$  tocopherol

curves when  $\alpha$  tocopherol was administered ( $P < .0001$ ) (Fig. 1, right panel).

#### 3.3. Surrogate markers of atherosclerotic risk and oxidative stress

The data showed similar baseline levels of each oxidative stress marker (oxy-LDL, GPx activity, and MDA) (Fig. 2)

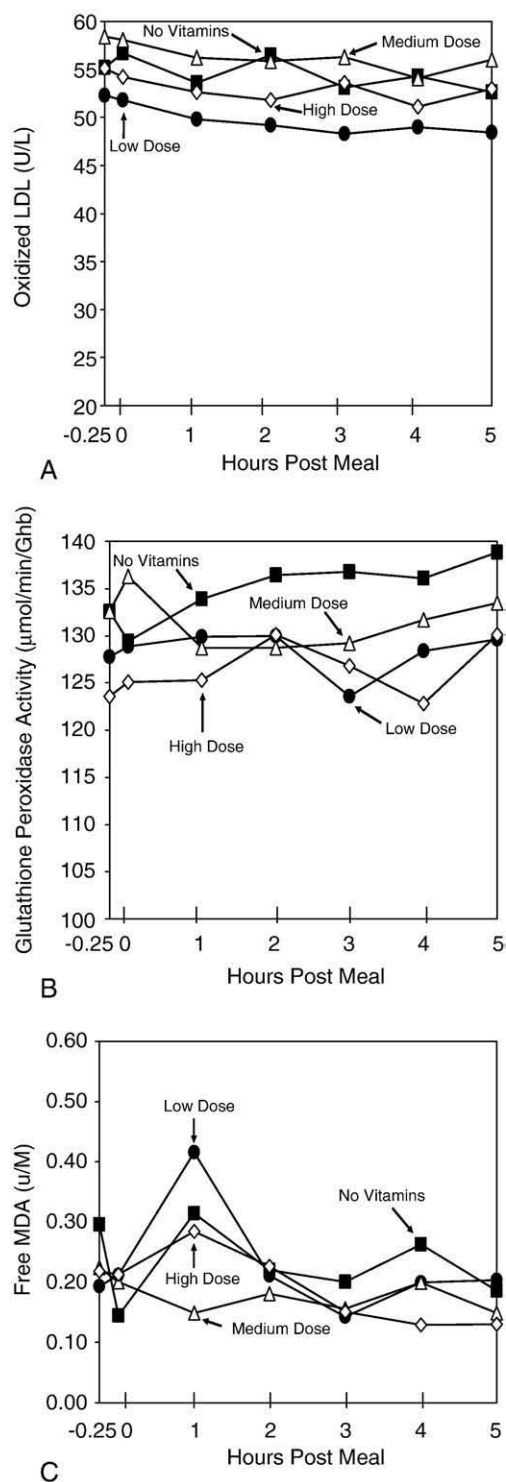


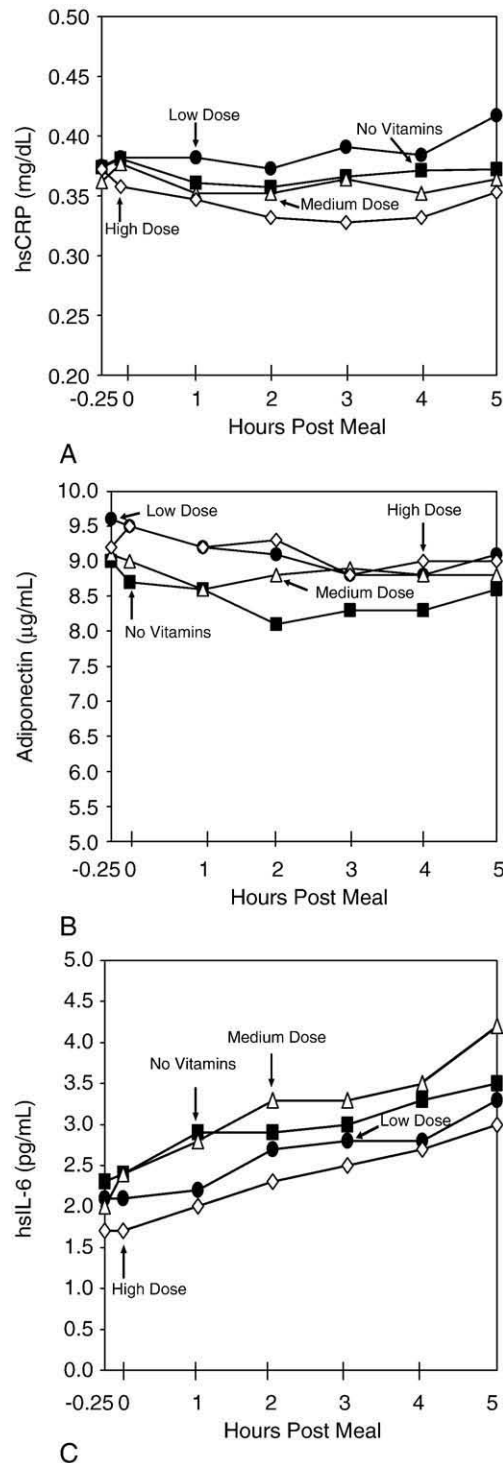
Fig. 2. Cardiovascular risk and oxidative stress markers. Plasma oxy-LDL (A), erythrocyte GPx activity (B), and plasma MDA (C) excursions during the no-vitamin arm (black square), low-dose vitamin arm (black circle), medium-dose vitamin arm (open triangle), and high-dose vitamin arm (open diamond). The range for the SEM for oxidized LDL at all data points was as follows: no-vitamin arm = 4.3 to 5.4, low-dose vitamin arm = 4.1 to 5.1, medium-dose vitamin arm = 4.5 to 5.9, and high-dose vitamin arm = 3.2 to 3.6. The range for the SEM for erythrocyte GPx activity at all data points was as follows: no-vitamin arm = 10.4 to 12.6, low-dose vitamin arm = 8.5 to 12.3, medium-dose vitamin arm = 10.0 to 12.1, and high-dose vitamin arm = 9.3 to 12.9. The range for the SEM for plasma MDA at all data points was as follows: no-vitamin arm = 0.026 to 0.112, low-dose vitamin arm = 0.037 to 0.331, medium-dose vitamin arm = 0.040 to 0.098, and high-dose vitamin arm = 0.032 to 0.166. For each of the markers of oxidative stress, no significant difference was observed between the different dosages of vitamin E supplementation ( $P > .05$ ).



across all study arms ( $P > .05$  for all). Each marker also had showed similar curves between each of its arms ( $P > .05$  for all) during the atherogenic high-fat meal.

### 3.4. Surrogate markers of inflammation

There were no significant differences seen in each of the baseline levels of CRP, adiponectin, and IL-6 (Fig. 3) during the various study conditions ( $P > .05$  for all). In addition, the



study arm curves for each marker also failed to show any significant variation between each other ( $P > .05$  for all).

### 3.5. Surrogate markers of hypercoagulation

Baseline levels of PAI-1 and fibrinogen (Fig. 4) were similar across all study arms ( $P > .05$ ). The PAI-1 levels displayed a significant test meal-induced fall in all study arms ( $P = .0004$ ), representing a normal diurnal decline. For both PAI-1 and fibrinogen, the placebo and vitamin arms showed similar baselines ( $P > .05$ ) and similar curves across time ( $P > .05$ ).

### 3.6. Other markers

Mean baseline levels of glucose, C-peptide, and insulin (Fig. 5) were similar for all study conditions ( $P > .05$  for all). Each marker showed a statistically significant postmeal elevation ( $P < .0001$  for all). There were no statistically significant differences seen between the curves of each study arm for each marker ( $P > .05$  for all).

For cholesterol and triglycerides, no significant differences were observed between mean baseline levels ( $P > .05$ ) and the study arm curves ( $P > .05$ ) (data not shown).

## 4. Discussion

This study demonstrated that daily supplementation of  $\alpha$  tocopherol plus vitamin C significantly reduced the circulating concentration of  $\gamma$  tocopherol. This suppression (by approximately 50%) was observed at all 3 dosages (low, medium, and high) of  $\alpha$  tocopherol. These dosages included all of the dosages used in clinical trials that observed or failed to observe a significant reduction in cardiovascular events [6]. In addition, at all dosages,  $\alpha$  tocopherol supplementation failed to lower surrogate markers of atherosclerotic risk and oxidative stress, inflammation, and hypercoagulation in a population with well-controlled type 2 diabetes mellitus. This observation was made despite the following: (1) use of a high-risk population with increased potential benefit from reduction of oxidative stress, (2) inclusion of a high-fat meal that has been shown to enhance

Fig. 3. Inflammation markers. Plasma hsCRP (A), adiponectin (B), and hsIL-6 (C) excursions during the no-vitamin arm (black square), low-dose vitamin arm (black circle), medium-dose vitamin arm (open triangle), and high-dose vitamin arm (open diamond). The range for the SEM for hsCRP at all data points was as follows: no-vitamin arm = 0.13 to 0.14, low-dose vitamin arm = 0.11 to 0.13, medium-dose vitamin arm = 0.10 to 0.11, and high-dose vitamin arm = 0.14 to 0.17. The range for the SEM for adiponectin at all data points was as follows: no-vitamin arm = 1.4 to 1.5, low-dose vitamin arm = 1.4 to 1.7, medium-dose vitamin arm = 1.4 to 1.6, and high-dose vitamin arm = 1.6 to 1.8. The range for the SEM for hsIL-6 at all data points was as follows: no-vitamin arm = 0.4 to 0.8, low-dose vitamin arm = 0.3 to 0.4, medium-dose vitamin arm = 0.2 to 0.6, and high-dose vitamin arm = 0.2 to 0.5. For each of the markers of inflammation, no significant difference was observed between the different dosages of vitamin E supplementation ( $P > .05$ ).

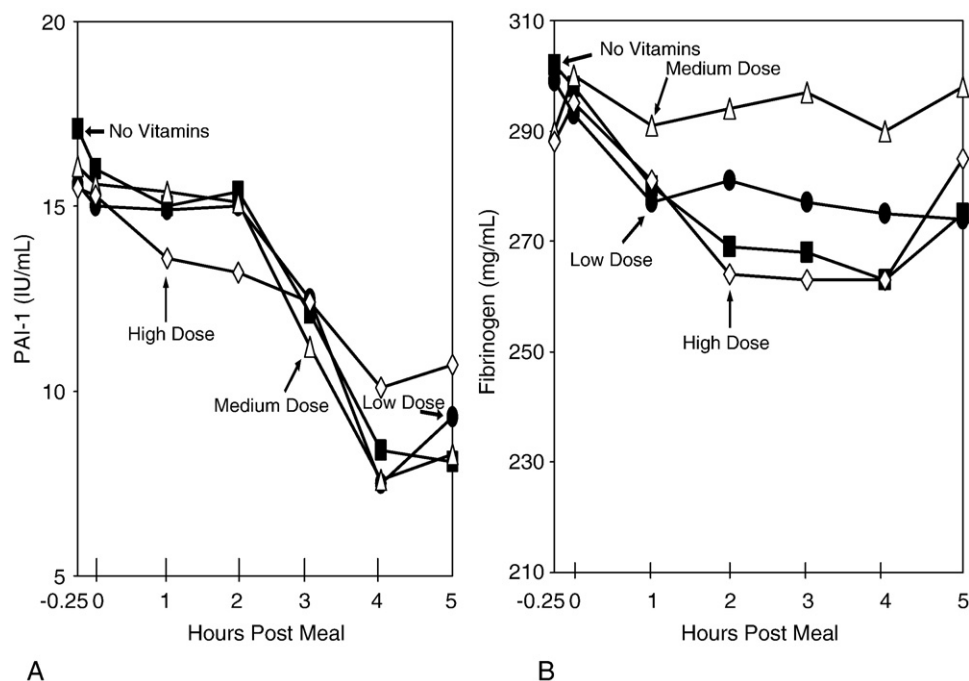


Fig. 4. Hypercoagulation markers. Plasma PAI-1 (A) and fibrinogen (B) excursions during the no-vitamin arm (black square), low-dose vitamin arm (black circle), medium-dose vitamin arm (open triangle), and high-dose vitamin arm (open diamond). The range for the SEM for PAI-1 at all data points was as follows: no-vitamin arm = 2.2 to 5.3, low-dose vitamin arm = 2.1 to 4.9, medium-dose vitamin arm = 2.7 to 5.7, and high-dose vitamin arm = 2.7 to 4.3. The range for the SEM for fibrinogen at all data points was as follows: no-vitamin arm = 19 to 24, low-dose vitamin arm = 12 to 19, medium-dose vitamin arm = 13 to 19, and high-dose vitamin arm = 18 to 24. For each of the markers of hypercoagulation, no significant difference was observed between the different dosages of vitamin E supplementation ( $P > .05$ ).

oxidative stress, (3) coadministration of vitamin C with vitamin E to replenish the vitamin E antioxidative radical, and (4) administration of both vitamins for 2 weeks before study. These vitamins were administered for 2 weeks and also several hours before the test meal to increase their efficacy in reducing inflammation and increasing fibrinolysis [38]. The bioavailability of oral vitamin E is greatest when administered with food [39], which is why the vitamins were given with a small breakfast (to ensure maximal absorption and hence antioxidant capacity). We gave natural RRR- $\alpha$ -tocopherol because it is twice as potent as the racemic synthetic  $\alpha$  tocopherol [40].

At high concentrations, vitamin C functions as an antioxidant that protects lipids from oxidative damage induced by aqueous peroxy radicals [10,11]. It also regenerates other antioxidants, such as flavonoids and glutathione. It may reverse endothelial dysfunction in peripheral arteries of individuals with high oxidative stress conditions, such as type 2 diabetes mellitus. Combined administration of vitamins E and C produces antioxidant effects primarily through the action of vitamin C elimination of the  $\alpha$  tocopherol radical. In vivo animal studies and in vitro studies show that vitamin C accomplishes this by exporting the radical away from a lipid medium into an aqueous medium [41,42]. The ability of vitamin C to preserve vitamin E in individuals who smoke has recently been demonstrated [43].

Type 2 diabetes mellitus individuals, when compared with healthy individuals, show greater oxidative stress in response to a standardized meal [44]. Hyperglycemia may increase free radical production through mechanisms of lipoprotein glycation, glucose autooxidation, and polyol pathway activation [44]. These individuals also have greater postprandial increments in triglyceride levels when compared with a healthy population. This leads to a postprandial lipidemia that causes increased oxidative stress and vascular dysfunction [45]. In general, populations with increased oxidative stress (eg, smokers, patients with CHD or end-stage renal disease, transplant recipients) have responded maximally to antioxidant vitamins [6,36,46,47].

The high-fat test meal, simulating a McDonald's Big Mac meal, was administered to enhance the level of oxidative stress. Previous studies have shown that a single high-fat meal transiently impairs endothelial function for at least 4 hours in healthy subjects and that oxidative stress is likely the mechanism because the administration of antioxidant vitamins E and C was able improve flow-mediated vasodilation [48,49]. Our high-fat meal used reheated cooking oil identical to that used in commercial fast food restaurants [50], which has been shown to contribute to decreased arterial endothelial-dependent vasodilation [51].

Choices of surrogate markers for this study were based on their roles in the atherosclerotic process. Oxidized LDL was measured because the oxidative conversion of LDL to oxy-

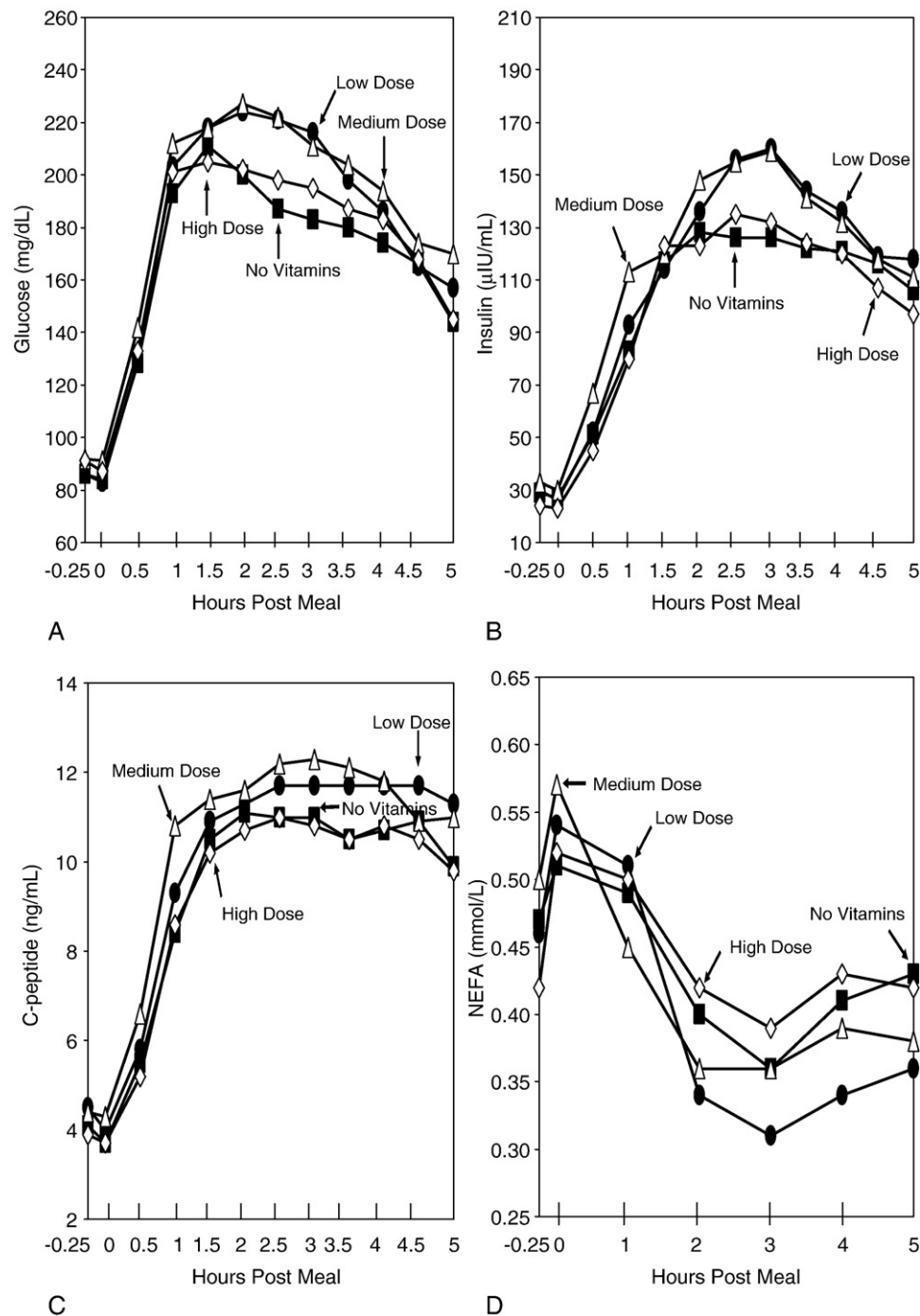


Fig. 5. Metabolic markers. Plasma glucose (A), insulin (B), C-peptide (C), and NEFA (D) excursions during the no-vitamin arm (black square), low-dose vitamin arm (black circle), medium-dose vitamin arm (open triangle), and high-dose vitamin arm (open diamond). The range for the SEM for glucose at all data points was as follows: no-vitamin arm = 4 to 17, low-dose vitamin arm = 5 to 21, medium-dose vitamin arm = 5 to 21, and high-dose vitamin arm = 5 to 17. The range for the SEM for insulin at all data points was as follows: no-vitamin arm = 8 to 26, low-dose vitamin arm = 7 to 40, medium-dose vitamin arm = 6 to 43, and high-dose vitamin arm = 5 to 40. The range for the SEM for C-peptide at all data points was as follows: no-vitamin arm = 0.5 to 1.1, low-dose vitamin arm = 0.5 to 1.2, medium-dose vitamin arm = 0.6 to 1.1, and high-dose vitamin arm = 0.5 to 1.0. The range for the SEM for NEFA at all data points was as follows: no-vitamin arm = 0.02 to 0.07, low-dose vitamin arm = 0.02 to 0.07, medium-dose vitamin arm = 0.03 to 0.05, and high-dose vitamin arm = 0.04 to 0.06. Although there was no significant difference between the different dosages of vitamin E supplementation and the metabolic markers ( $P > .05$ ), the metabolic effects of the high fat are evident.

LDL is a key event in the development of the fatty streak and because oxy-LDL is more atherogenic than its native form [52]. The antioxidant enzyme glutathione peroxidase-1 has a

central role in preventing the development of reactive oxygen species, and low levels of this enzyme are associated with increased risk of cardiovascular events [24]. Our study

measured GPx activity, but it should be noted that glutathione is affected by not only GPx activity but also GPx reductase activity as well as glutathione synthase. Although GPx activity was unchanged in our study, it is possible that glutathione levels could have been reduced in patients with diabetes [53]. Additional studies will be required to determine the actual effects of  $\alpha$  vitamin E and vitamin C on glutathione. Malondialdehyde is a by-product of lipid peroxidation in atherosclerosis [25]. Nonesterified fatty acids (free fatty acids) increase insulin resistance and serve as a substrate for hepatic triglyceride production [54]. C-reactive protein has a proatherogenic role in vascular smooth muscle cells, monocyte macrophages, and endothelial cells; and elevated levels of CRP predict a greater risk of cardiovascular disease [55]. Adiponectin has been shown to increase fatty acid oxidation and glucose uptake, reduce fatty acid synthesis, and reduce molecules involved in gluconeogenesis [56]. Low adiponectin levels are found in obese and diabetic individuals, and high levels are associated with lower atherosclerotic risk of myocardial infarction in men [57]. The proinflammatory response involves the secretion of the cytokine IL-6 from macrophages, which ultimately stimulates the secretion of CRP [55]. Plasminogen activator inhibitor-1 is a suppressor of fibrinolysis through the inhibition of tissue plasminogen activator. Increased PAI-1 activity has been demonstrated in people with coronary artery disease, and elevated PAI-1 is a strong risk factor for cardiovascular disease [58]. Fibrinogen, which is a product of the coagulation cascade, is also a risk factor for cardiovascular disease [31].

Our study's observation that  $\alpha$  tocopherol supplementation had no effect on surrogate atherosclerotic markers is best explained by its most important finding: low, medium, and high doses of  $\alpha$  tocopherol plus vitamin C suppressed circulating levels of  $\gamma$  tocopherol below those observed during the placebo arm of the study.  $\gamma$  Tocopherol constitutes 70% to 80% of the vitamin E in an average American diet. It is especially abundant in nuts, vegetable oils, and plant seeds [59,60]. Compared with  $\alpha$  tocopherol, it has a much lower concentration in the human body but much greater bioactivity.

Recent studies have made strong arguments for the unique benefits of  $\gamma$  tocopherol. Saldeen et al [61] showed that both  $\alpha$  and  $\gamma$  tocopherol decreased platelet aggregation and slowed arterial thrombus formation in rats. They also reduced LDL oxidation and lipid peroxidation, reduced superoxide generation, and increased superoxide dismutase activity. However, in all of these effects,  $\gamma$  tocopherol was significantly more potent than  $\alpha$  tocopherol [61]. In addition,  $\gamma$  tocopherol and its metabolite have unique anti-inflammatory properties [62] and detoxify reactive nitrogen oxide species in a manner superior to  $\alpha$  tocopherol [63]. In regard to human in vivo studies, 2 recent case-control trials showed lower concentrations of  $\gamma$  tocopherol in subjects with CHD when compared with a healthy control group [64,65]. One of these studies noted that these changes were not seen with  $\alpha$  tocopherol [64].

Large epidemiologic studies have shown that vitamin E from food (which is mostly  $\gamma$  tocopherol) is inversely associated with the risk of death from CHD and stroke, whereas vitamin E from supplements (mainly  $\alpha$  tocopherol) showed no benefits [66,67]. Both  $\alpha$  and  $\gamma$  tocopherol compete for the hepatic  $\alpha$  tocopherol transfer protein, which preferentially binds to  $\gamma$  tocopherol [68,69]. The degree of binding to this protein determines the biological activity of the vitamin E molecule [69]. This may explain why  $\alpha$  tocopherol supplementation reduces  $\gamma$  tocopherol levels [70]. It is interesting that  $\alpha$  vitamin E, even at the lowest replacement dose, significantly suppressed  $\gamma$  vitamin E. This indicates that this suppression is very sensitive to low circulating levels of  $\alpha$  vitamin E. The finding that vitamin E and vitamin C supplements failed to worsen the indices of oxidative stress and inflammation despite suppressing the circulating levels of  $\gamma$  vitamin E may indicate that there may not be a major effect of a 50% decrease in  $\gamma$  tocopherol.

A potential limitation of this study is that there was no washout scheduled between each change in vitamin E dosage. A washout period was omitted because the entire study for any individual required 2 months to complete, and we did not want to prolong the study for that individual because of the potential change in diet and because negative adherence might occur. Because each of the treatment groups was randomized, this may have taken into account any problem with washout that may have existed. In addition, an alternative interpretation of our results is that the lack of effect on various biomarkers might result from the combined effects of increased  $\alpha$  vitamin E and decreased  $\gamma$  vitamin E or from a compensatory effect of  $\alpha$  vitamin E in the presence of reduced concentrations of  $\gamma$  vitamin E.

Our study demonstrates that low-, medium-, and high-dose  $\alpha$  tocopherol plus vitamin C had no effect on markers of atherosclerosis, when compared with placebo, in a high-risk population. This is best explained by the suppression of the powerful antioxidant  $\gamma$  tocopherol via  $\alpha$  tocopherol supplementation. A recent study [71] demonstrates the superiority of  $\gamma$  vitamin E over  $\alpha$  vitamin E in improving oxidative stress in patients with the metabolic syndrome. No effect was observed when only  $\alpha$  vitamin E was administered, whereas a partial response was observed when both  $\alpha$  and  $\gamma$  vitamin E were given to the patients. The maximal response was observed when only purified vitamin E was given. This is likely to be one mechanism to explain the results of several recent large clinical trials demonstrating failure of vitamin E supplementation to improve cardiovascular outcomes [6–9]. A recent publication has indicated that high doses of vitamin C increases the vitamin E content of LDL in type 2 diabetes mellitus [72]. Our study does not directly address this possibility, although it may have contributed to the suppression of  $\gamma$  vitamin E after the increasing doses of  $\alpha$  vitamin E plus vitamin C in our protocol. Additional studies will be required to separate out the effects of  $\alpha$  vitamin E and vitamin C on the suppression of  $\gamma$  vitamin E. Because low-dose  $\alpha$  tocopherol is an ineffective antioxidant and doses



greater than 400 IU/d may increase all-cause mortality [6], we do not recommend the use of these supplements on a daily basis.

## Acknowledgment

This research was supported by the University of New Mexico GCRC (National Institutes of Health National Center for Research Resources GCRC grant 5 M01-RR00997) and by the American Diabetes Association grant 7-04-CR-41.

## References

- [1] Neuzil J, Weber C, Kontush A. The role of Vitamin E in atherogenesis: linking the chemical, biological and clinical aspects of the disease. *Atherosclerosis* 2001;157:257-83.
- [2] Jiang Q, Christen S, Shigenaga M, Ames BN.  $\gamma$ -Tocopherol, the major form of vitamin E in the US diet, deserves more attention. *Am J Clin Nutr* 2001;74:714-22.
- [3] Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* 1993;328:1450-6.
- [4] Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary disease in women. *N Engl J Med* 1993;328:1444-9.
- [5] Pruthi S, Allison TG, Hensrud D. Vitamin E supplementation in the prevention of coronary heart disease. *Mayo Clin Proc* 2001;76:1131-6.
- [6] Miller III ER, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 2005;142:37-46.
- [7] Lee IM, Cook NR, Gaziano JM, Gordon D, Ridker PM, Manson JE, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer. The Women's Health Study: a randomized controlled trial. *JAMA* 2005;294:56-65.
- [8] Lonn E, Bosch J, Sheridan P, Pogue J, Arnold JM, et al. The HOPE and HOPE-TOO trial investigators. Effects of long-term vitamin E supplementation on cardiovascular events and cancer. *JAMA* 2005;293:1338-47.
- [9] Yusuf S, Dagenais G, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 2000;342:154-60.
- [10] Frei B. Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage. *Am J Clin Nutr* 1991;54:1113S-8S.
- [11] Niki E. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. *Am J Clin Nutr* 1991;54:1119S-24S.
- [12] Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest* 1996;97:22-8.
- [13] Carr A, Zhu BZ, Frei B. Potential antiatherogenic mechanisms of ascorbate (vitamin C) and  $\alpha$ -tocopherol (vitamin E). *Circ Res* 2000;87:349-54.
- [14] Fang JC, Kinlay S, Beltrame J, Hikiti H, Wainstein M, Behrendt D, et al. Effects of vitamins C and E on the progression of transplant-associated arteriosclerosis: a randomised trial. *Lancet* 2002;359:1108-13.
- [15] Salonen JT, Nyyssonen K, Salonen R, Lakka HM, Kaikkonen J, Porkkala-Sarataho E, et al. Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis. *J Intern Med* 2000;248:377-86.
- [16] Salonen RM, Nyyssonen K, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Rissanen TH, et al. Six-year effect of combined vitamin C and E supplementation on atherosclerotic program: the Antioxidant Supplementation in Atherosclerosis Prevention study (ASAP). *Circulation* 2003;107:947-53.
- [17] Boaz M, Smetana S, Weinstein T, Matas Z, Gafter U, Iaina A, et al. Secondary Prevention with Antioxidants of Cardiovascular disease in Endstage renal disease (SPACE): randomized placebo-controlled trial. *Lancet* 2000;356:1213-8.
- [18] Huang H, Appel LJ. Supplementation of diets with alpha-tocopherol reduces serum concentrations of  $\gamma$ - and  $\delta$ -tocopherol in humans. *J Nutr* 2003;133:3137-40.
- [19] Handelman GJ, Machlin LJ, Fitch K, Weiter JJ, Dratz EA. Oral alpha-tocopherol supplements decrease plasma gamma-tocopherol. *J Nutr* 1985;115:807-13.
- [20] Thurnham DI, Davies JA, Crump BJ, Situnayake RD, Davis M. The use of different lipids to express serum tocopherol: lipid ratios for the measurement of vitamin E status. *Ann Clin Biochem* 1986;23:514-20.
- [21] Hansen LG, Warwick WJ. An improved assay method for serum vitamins A and E using fluorometry. *Am J Clin Pathol* 1978;70:922-3.
- [22] Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 1998;98:1487-94.
- [23] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;70:158-69.
- [24] Blankenberg S, Rupprecht HJ, Bickel C, Torzewski M, Hafner G, Tiret L, et al. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. *N Eng J Med* 2003;349:1605-13.
- [25] Yeo HC, Helbock HJ, Chyu DW, Ames BN. Assay of malondialdehyde in biological fluids by gas chromatography-mass spectrometry. *Anal Biochem* 1994;220:391-6.
- [26] Elphick MC. Modified colorimetric ultramicro method for estimating NEFA in serum. *J Clin Pathol* 1968;21:567-70.
- [27] Roberts WL, Moulton L, Law TC, Farrow G, Cooper-Anderson M, Savory J, et al. Evaluation of nine automated high-sensitivity C-reactive protein methods: implications for clinical and epidemiological applications. Part 2. *Clin Chem* 2001;47:418-25.
- [28] Suominen P. Evaluation of an enzyme immunometric assay to measure serum adiponectin concentrations. *Clin Chem* 2004;50:219-21.
- [29] Moody MD, Van Arsdell SW, Murphy KP, Orencole SF, Burns C. Array-based ELISAs for high-throughput analysis of human cytokines. *Biotechniques* 2001;31:186-90,192-94.
- [30] Declerck PJ, Alessi MC, Verstreken M, Kruithof ED, Juhan-Vague I, Collen D. Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. *Blood* 1988;71:220-5.
- [31] Geffken DF, Keating FG, Cornell ES, Bovill EG, Tracy RP. The measurement of fibrinogen in population-based research. Studies on instrumentation and methodology. *Arch Pathol Lab Med* 1994;118:1106-9.
- [32] Bondar RJ, Mead DC. Evaluation of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* in hexokinase method for determining glucose in serum. *Clin Chem* 1974;20:586-90.
- [33] Hardy RW, Cohn M, Konrad RJ. Automated chemiluminescent assay for C-peptide. *J Clin Lab Anal* 2000;14:17-9.
- [34] Andersen L, Dinesen B, Jorgensen PN, Poulsen F, Roder ME. Enzyme immunoassay for intact insulin in serum or plasma. *Clin Chem* 1993;39:578-82.
- [35] Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem* 1982;28:2077-80.
- [36] Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem* 1974;20:470-5.

- [37] Handelman GJ, Machlin LJ, Fitch K, Weiter JJ, Dratz EA. Oral alpha-tocopherol supplements decrease plasma gamma tocopherol levels in humans. *J Nutr* 1985;348:241–51.
- [38] Carroll MF, Schade DS. Timing of antioxidant vitamin ingestion alters postprandial proatherogenic serum markers. *Circulation* 2003;108:24–31.
- [39] Iuliano L, Micheletta F, Maranghi M, Frati G, Dieczfalusy U, Violi F. Bioavailability of vitamin E as function of food intake in healthy subjects. *Arterioscler Thromb Vasc Biol* 2001;21:e34–7.
- [40] Brigelius-Flohe R, Traber M. Vitamin E: function and metabolism. *FASEB J* 1999;13:1145–55.
- [41] Neuzil J, Thomas SR, Stocker R. Requirement for, promotion, or inhibition by  $\alpha$ -tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation. *Free Radic Biol Med* 1997;22:57–71.
- [42] Suarna C, Dean RT, May J, Stocker R. Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol* 1995;15:1616–24.
- [43] Bruno RS, Leonard SW, Atkinson J, Montine TJ, Ramakrishnan R, Bray TM, et al. Faster plasma vitamin E disappearance in smokers is normalized by vitamin C supplementation. *Free Radic Biol Med* 2006;40:689–97.
- [44] Ceriello A, Bortolotti N, Motz E, Crescentini A, Lizzio S, Russo A, et al. Meal-generated oxidative stress in type 2 diabetic patients. *Diabetes Care* 1998;21:1529–33.
- [45] Nappo F, Esposito K, Cioffi M, Giugliano G, Molinari AM, Paolisso G, et al. Postprandial endothelial activation in healthy subjects in type 2 diabetic patients: role of fat and carbohydrate meals. *J Am Coll of Cardiol* 2002;39:1145–50.
- [46] Liu L, Meydani M, Mayer J. Combined vitamin C and E supplementation retards early progression of arteriosclerosis in heart transplant patients. *Nutr Rev* 2002;60:368–71.
- [47] Steinberg D, Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? *Circulation* 2002;105:2107–11.
- [48] Plotnick GD, Corretti MC, Vogel RA. Effect of antioxidant vitamins on the transient impairment of endothelium-dependent brachial artery vasoactivity following a single high-fat meal. *JAMA* 1997;278:1682–6.
- [49] Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. *Am J Cardiol* 1997;79:350–4.
- [50] McSavage J, Trevisan S. The use and abuse of frying oil. *Food Serv technol* 2001;1:85–92.
- [51] Williams MJ, Sutherland WH, McCormick MP, de Jong SA, Walker RJ, Wilkins GT. Impaired endothelial function following a meal rich in used cooking fat. *J Am Coll Cardiol* 1999;33:1050–5.
- [52] Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 1991;88:1785–92.
- [53] Murakami K, Kondo T, Ohtsuka Y, Fujiwara Y, Shimada M, Kawakami Y. Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus. *Metabolism* 1989;38:753–8.
- [54] McGarry JD. Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 2002;51:7–18.
- [55] Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14,719 initially healthy American women. *JAMA* 2005;294:56–65.
- [56] Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002;8:1288–95.
- [57] Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, Rimm EB. Plasma adiponectin levels and risk of myocardial infarction in men. *JAMA* 2004;291:1730–6.
- [58] Devaraj S, Chan Jr AV, Jialal I. Alpha-tocopherol supplementation decreases plasminogen activator inhibitor-1 and P-selectin levels in type 2 diabetic patients. *Diabetes Care* 2002;25:524–9.
- [59] Lehmann J, Martin HL, Lashley EL, Marshall MW, Judd JT. Vitamin E in foods from high and low linoleic acid diets. *J Am Diet Assoc* 1986;86:1208–16.
- [60] Bieri JC, Everts RP. Tocopherols and fatty acids in American diets. *J Am Diet Assoc* 1973;62:147–51.
- [61] Saldeen T, Li D, Mehta JL. Differential effects of  $\alpha$ - and  $\gamma$ -tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation, and arterial thrombogenesis. *J Am Coll Cardiol* 1999;34:1208–15.
- [62] Jiang Q, Elson-Schwab I, Courtemanche C, Ames BN.  $\gamma$ -Tocopherol and its major metabolite, in contrast to  $\alpha$ -tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proc Natl Acad Sci U S A* 2000;97:11494–9.
- [63] Cooney RV, Franke AA, Harwood PJ, Hatch-Pigott V, Custer LJ, Mordan LJ. Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol. *Proc Natl Acad Sci U S A* 1993;90:1771–5.
- [64] Ohrvall M, Sundlof G, Vessby B. Gamma, but not alpha, tocopherol levels in serum are reduced in coronary heart disease patients. *J Intern Med* 1996;239:111–7.
- [65] Kontush A, Spranger T, Reich A, Baum K, Beisiegel U. Lipophilic antioxidants in blood plasma as markers of atherosclerosis: the role of alpha-carotene and gamma-tocopherol. *Atherosclerosis* 1999;144:117–22.
- [66] Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *N Eng J Med* 1996;334:1156–62.
- [67] Yochum LA, Folsom AR, Kushi LH. Intake of antioxidant vitamins and risk of death from stroke in postmenopausal women. *Am J Clin Nutr* 2000;72:476–83.
- [68] Hosomi A, Arita M, Sato Y, Kiyose C, Ueda T, Igarashi O, et al. Affinity for  $\alpha$ -tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. *FEBS Letters* 1997;409:105–8.
- [69] Catignani GL, Bieri JG. Rat liver alpha-tocopherol binding protein. *Biochim Biophys Acta* 1977;497:349–57.
- [70] Robinson I, Gonzalez de Serna D, Gutierrez A, Schade DS. Vitamin E in man—an explanation of clinical trial failure. *Endocr Pract* 2006;12:576–82.
- [71] Devaraj S, Leonard S, Trabert MG, Jialal I. Gamma-tocopherol supplementation alone and in combination with alpha-tocopherol alters biomarkers of oxidative stress and inflammation in subjects with metabolic syndrome. *Free Radic Biol Med* 2008;44:1203–8.
- [72] Tessier D, Khalil A, Trottier L, Fülöp T. Effects of vitamin C supplementation on antioxidants and lipid peroxidation markers in elderly subjects with type 2 diabetes. *Arch Geront Geriatr* 2008, doi: 10.1016/i.archger.2007.10.005.